Original article

Relationship between receptors for insulin-like growth factor – I, steroid hormones and apoptosis-associated proteins in canine mammary tumors

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Abstract

In the veterinary literature there are few data concerning the expression of insulin-like growth factor type I (IGF-IR) in the canine mammary gland tumors. The aim of the present study was the evaluation of IGF-IR expression and its correlation to the expression of estrogen receptor α (ERα) and progesterone receptor (PR), proteins: Bcl-2, Bax, p53 in canine mammary gland tumors, and also a correlation with other features: bitch's age, tumor diameter, histologic type of tumor, degree of histologic malignancy, proliferate activity. The study was done on 112 epithelial neoplasms: 21 (19%) were adenoma, 38 (34%) complex carcinoma (adenocarcinoma), 47 (42%) simple carcinoma (adenocarcinoma) and 6 (5%) solid carcinoma. Histochemistry and immunohistochemistry methods were employed. It was shown that more common and/or higher IGF-IR expression in cells of canine mammary gland tumors was related to the histologic type of cancer of worse prognostic (solid and simple carcinoma), high histologic degree of malignancy (III°) but the statistical analysis did not reveal any significant differences. We observed the high degree of IGF-IR expression in tumors which displayed the high ERα and PR expression. These results suggest the involvement of IGF-IR in the development of hormonosensitive canine mammary tumors. Additionally, the significant positive correlation between expression of IGF-IR and p53, Bax was found. Our study provides some evidence that interactions exist between the IGF-IR and these apoptosis-associated proteins may contribute to the development and progression of canine mammary gland tumors. These results require further investigations.

Key words: neoplasms, dogs, mammary gland, IGF-IR, hormone steroid receptors

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Introduction

There are many hypotheses formed in an attempt to explain the etiopathogenesis of the canine mammary gland tumors. Most of them underline the significant role of hormonal agents. In recent years new pieces of the puzzle have been added, that are related to auto- or paracrine action, which can stimulate cells growth. This kind of activity is demonstrated for growth factors (Dickson et al. 1995, Oosterlaken-Dijksterhuis et al. 1999, Gama et al. 2009). According to the research concerning the breast cancer in women it emerges that insulin-like growth factor type I (IGF-I) increases cellular proliferation, which can induce neoplasm formation.

The family of insulin-like growth factors includes two peptides: IGF-I (also known as somatomedin C) and IGF-II, which by their receptors influence the cells. Insulin-like growth factor type I receptor (IGF-IR) is a heterotetramer structured from two outside membrane subunits α, that constitute a binding site for the growth factor, and two subunits β, which exhibit tyrosine kinase activity and take part in the initiation of intracellular signaling pathways of mitogenic signals. IGF-I also regulates intracellular antiapoptotic proteins, including Bcl-2 protein, survivin, cIAP-2. Particularly interesting are studies that confirmed the presence of the synergistic activity of estrogen/estrogen receptor type α (ERα) and IGF-I/IGF-IR pathway (so called cross-talk) in the breast cancer cells *in vitro* and *in vivo*, and their potential prognostic value (Yee et al. 2000, Bradley et al. 2008). In the veterinary medicine, papers on IGF-I role in the canine mammary gland tumors are less common and up to now there is no data dealing with determination of IGF-IR expression in these type of cancers.

The aim of the present study was the evaluation of IGF-IR expression in canine mammary gland tumors, and definition of its correlation with the expression of estrogen receptor α (ERα), progesterone receptor (PR) and proteins: Bcl-2, Bax, p53, and with other characteristics as followed: bitch's age, tumor size, histologic type, the degree of histologic malignancy as well as proliferative activity.

Materials and Methods

The study material consisted of 112 canine mammary gland tumors. Tumor specimens were fixed in phosphate buffered 10% formalin. Subsequently tumor samples were dehydrated in alcohol dilution series, xylen cleared, and embedded in paraffin. Paraffin blocks were sliced into 4 μm sections, which were hematoxylin-eosin stained. In the sections following parameters were determined:

– histologic type of the tumor, based on WHO classification (Misdorp 2002),

– mitotic index (MI) as a mean number of mitosis in 10 vision fields, at the objective magnification 40x (Dutra et al. 2008),

– degree of histologic malignancy, according to the rules elaborated by Misdorp (2002).

For immunohistochemistry (IHC), paraffin embedded sections were sticked to glass slide covered with 2% Silan solution in acetone. After deparaffining and rehydrating in reverted alcohol dilution series the sections were boiled in citrate buffer of pH 6.0 in a microwave oven. After cooling, the sections were placed in 3% perhydrol, then washed twice in distilled water, and placed in TRIS buffer (pH 7.4). Subsequently appropriate monoclonal or polyclonal antibodies were added in suitable dilutions (Table 1) and incubated in a humidified chamber for 60 minutes. Then, EnVision+System-HRP (Dako) was employed to visualize the sections. In further steps the sections were washed in TRIS buffer and chromogen solution, prepared according to the manufacturers instructions (Dako), was dropped. In a final stage, the sections were carried along the alcohol series of increasing concentrations, xylen exposed and mounted with DPX medium (Gurr®). As a positive control specimens of dog's uterus and lymph nodes and known positive tissue specimens from canine mammary tumors were used.

Immunohistochemistry evaluation was done with a light microscope at the magnification 40x. The interpretation of the results for immunochemistry reaction for Ki-67, p53 protein, ERα, PR was according to the following: positively stained nuclei of neoplastic cells

Table 1. The antibodies used for immunohistochemical analysis.

Antibody	Source	Specificity	Working dilution
Monoclonal, Mouse Anti-Human Ki-67 Antigen	Dako	Ki-67	1/75
Polyclonal, Rabbit Anti-Human p53 protein	SantaCruz Biotech.	p53	1/50
Monoclonal, Mouse Anti-Human Estrogen Receptor α	Dako,	ERh	1/35
Monoclonal Mouse Anti-Human Progesteron Receptor	Immunotech,	PR	ready-to-use
Polyclonal, Rabbit Anti-Human Insulin-like growth factor receptor type 1	SantaCruz Biotech.	IGF-IR	1/50
Polyclonal, Rabbit Anti-Human Bcl-2 protein	SantaCruz Biotech.	Bcl-2	1/50
Polyclonal, Rabbit Anti-Human Bax protein	SantaCruz Biotech.	Bax	1/50

Table 2. Interpretation of results of immunohistochemical staining for IGF-IR, Bcl-2, Bax.

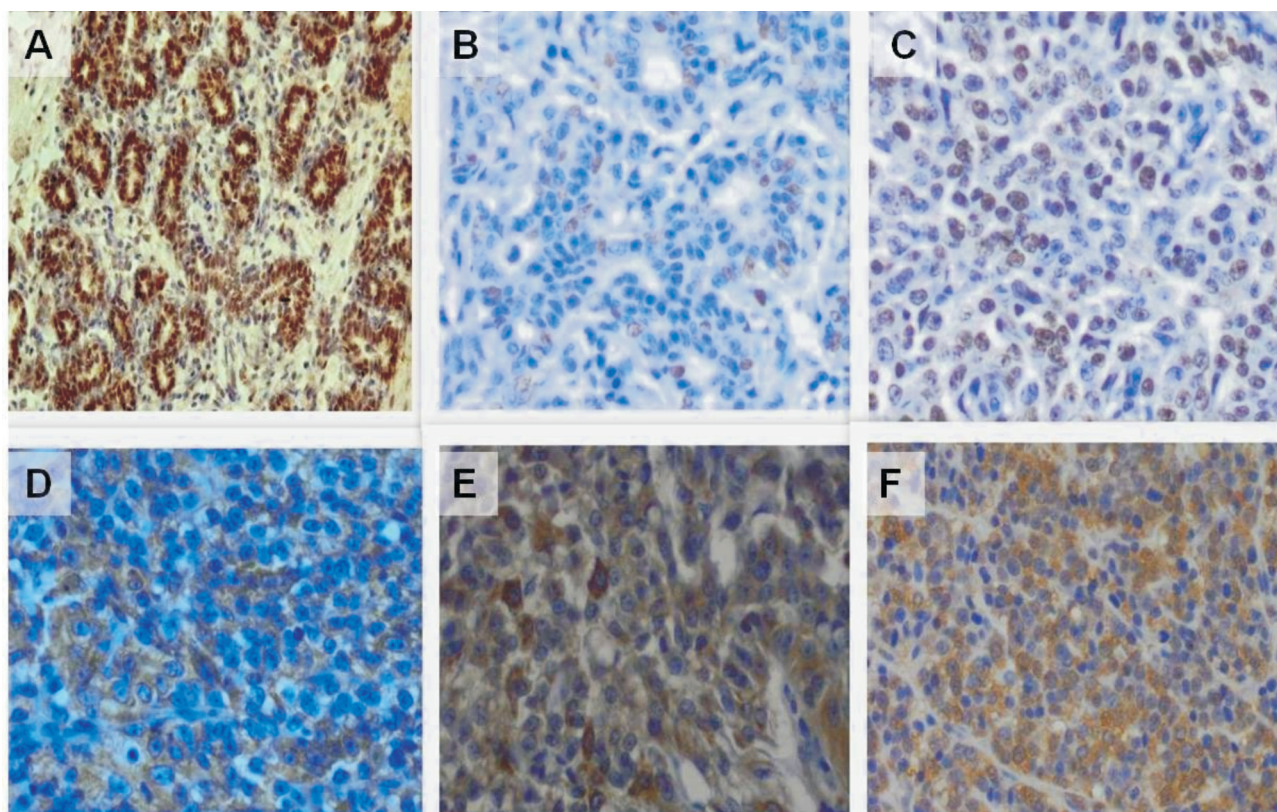| Degree of expression	IGF-IR	Bcl-2, Bax
0	Negative staining	Negative staining
1st	< 10% positive neoplastic cells	< 25% positive neoplastic cells
2nd	10-50% positive neoplastic cells	25-75% positive neoplastic cells
3rd	> 50% positive neoplastic cells	> 75% positive neoplastic cells

Fig. 1. Immunohistochemical results. Positive cells were stained brown: A – ERα(+). Simple carcinoma, B – PR(+). Simple carcinoma, C – p53(+). Solid carcinoma, D – IGF-IR(+). Solid carcinoma, E – Bcl-2(+). Complex carcinoma, F – Bax(+). Solid carcinoma. IHC method, original magnifications: A, 100x, B-F. Magnification x400

were count in 1 000 of neoplastic cells, and presented as an arithmetic mean of all calculations, according to the method proposed by Nieto et al. (2000), Millanta et al. (2005) and Rodo et al. (2008). In case of IGF-IR, Bcl-2, Bax additionally to the results' interpretation, a scale that correlates to the expression degree was employed (Table 2). In case of Bax protein expression, positively stained cells' cytoplasm was evaluated, and in several tumors color reaction in nuclei as well (Vakkala et al. 1999, Koda et al. 2005a, Aggarwal et al. 2007, Maor et al. 2007).

The results were analyzed with the aim of statistic software SPSS 14.0. Multiple comparisons between independent features were calculated by Kruskal-Wallis test. For correlations between two independent features Mann-Whitney U test was employed. Chi-square test was used to compare the distribution of the characteristics investigated. Correlation between features was established based on Spearman's test. The results were presented as arithmetic means and standard errors of the mean (mean value and SEM). The differences with $p \leq 0.05$ were regarded as statistically significant.

Results

Among 112 of the tumors that originated from the epithelium 21 (19%) were adenoma, 38 (34%) complex carcinoma, 47 (42%) simple carcinoma and 6 (5%) solid carcinoma. The material tested originated from bitches representing 22 breeds (among others the most common were: dachshund – 18%, German shepherd
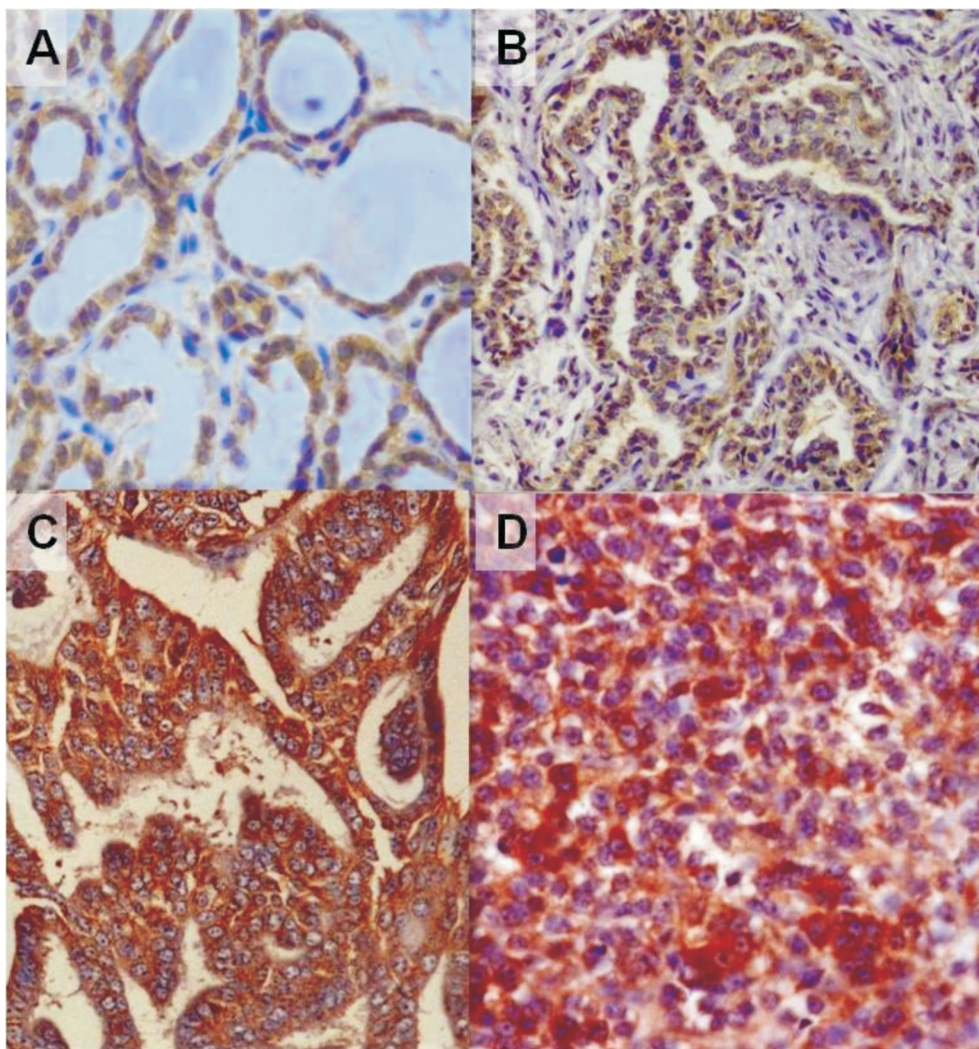
Fig. 2. Representative pictures of IGF-IR expression in canine mammary tumors. A. adenoma, B. complex carcinoma, C. simple carcinoma, D. solid carcinoma. IHC method, original magnifications: A, C, D magnification x400, B x200.

– 11% and mongrels – 30%). Mean age was 9.7 years, and mean diameter of the tumors studied was 4.6 cm. ERh expression was shown in 36% of the cancer cells nuclei tested, while PR in 69% ones. The color reaction in the cytoplasm of neoplastic cells was considered positive for IGF-IR in 67% and for Bcl-2 in 72% of the tumors tested. The accumulation of p53 protein was revealed in nuclei of 38% of tumors, while Bax protein expression in 54% of cells. The results of the immunohistochemistry reaction are presented in Figs. 1 and 2.

The analysis did not reveal any statistically significant differences between the mean number of cells featuring positive reaction to ERα, PR, Bcl-2, IGF-IR as well as between the percentage of positive cases showed ERα, PR, Bcl-2, IGF-IR expression and bitch's age, tumor size, histologic type and histologic degree of malignancy. It is noteworthy that IGF-IR expression most commonly was displayed in solid (100%) and simple carcinomas (72%). In an assessment of the

mean cell number showing IGF-IR expression, also higher expression was observed in simple and solid carcinomas. In a study of relationship of IGF-IR expression degree and type of neoplasm, and a degree of histologic malignancy, it was shown that the proportion of tumors with the 3rd degree of IGF-IR expression was higher in solid carcinomas (33.3%) and simple carcinomas (29.8%) as compared to complex carcinomas (10.5%), while the most of the tumors with 0 degree of IGF-IR expression belonged to complex carcinomas (42.1%), adenomas (38.2%), and in I^o and II° of histologic malignancy carcinomas (32.4% and 34.8%, respectively).

The study on the relationship between steroid hormone receptors and Ki-67 antigen expression in tumors of various degree of IGF-IR expression revealed significant differences in case of estrogen receptor α (p=0.001). The results are presented in Fig. 3. There was a positive correlation between IGF-IR and p53, Bax protein expression (respectively r=0.210,

p=0.027 and r=0.371, p=0.000). Among simple carcinomas (n=47) the highest expression of IGF-IR was demonstrated in case of the highest expression of PR (r=0.293, p=0.043).
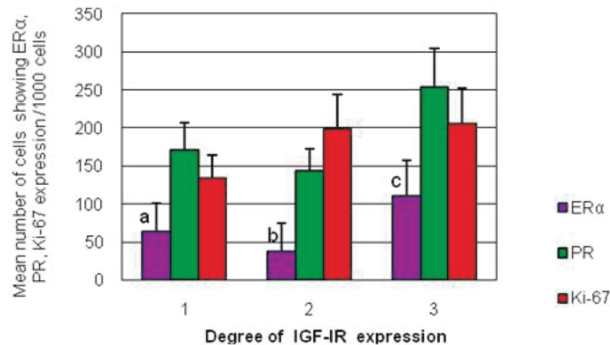

Fig. 3. The mean number of neoplastic cells showing the expression of ERα, PR, Ki-67 in relation to the degree of IGF-IR expression. Higher activity of proliferation assessed by Ki-67 expression was shown in tumors of the 2nd and the 3rd degree of IGF-IR expression, but there were no significant differences found between these groups. Increased expression of ERα and PR was observed in tumors with a high degree of expression of IGF-IR (significant differences were found only in case of ERα). Results presented as a mean value and SEM. Means marked with different letters are statistically significantly different (p≤0.05). Kruskal-Wallis test was employed in statistical analysis.

The expression of Ki-67, MI, p53 and Bax proteins appeared to be significantly dependent on the type (respectively for Ki-67: p=0.001 and for all factors: p=0.000) and histologic malignancy degree of the tumor (respectively for Ki-67, MI, p53: p=0.000 and for Bax p=0.016). Positive p53 reaction was observed in 53% cases of simple carcinomas, 32% cases of complex carcinomas and in all (100% cases) solid carcinomas tested, but was absent from adenomas. Positive correlation between p53 and MI, Ki-67 was observed (r=0.587, p=0.000 and r=0.342, p=0.000, respectively).

Discussion

In recent years a number of papers emerged, that indicate a potential role of IGF-I/IGF-IR in the etiopathogenesis of the breast cancer in women, and as well in canine mammary gland tumors (Oosterlaken-Dijkstrehuis et al. 1999, Koda et al. 2003, Mauro et al. 2003, Queiroga et al. 2008). In papers concerning the canine mammary gland tumors no information regarding detection of IGF-IR expression by IHC method can be found. In some immunohistochemistry studies Shimizu et al. (2004) and Koda et al. (2005a) showed positive cytoplasmatic reaction for IGF-IR in 43.8% and 56% of primary breast cancers, respectively,

while Happerfield et al. (1997) found it in 90% of carcinomas. However, the expression of IGF-IR was identified only in the cytoplasm of neoplastic cells (in 21% of cases) and in both cytoplasm and cell membranes (in 64% of cases). The present study demonstrated that 67% of all tumors displayed the IGF-IR expression in cytoplasm of neoplastic cells. Similarly to study concerning the breast cancer in women, we did not demonstrate the dependence between IGF-IR expression and animals' age, tumor size, type and the degree of histologic malignancy (Papa et al. 1993, Shimizu et al. 2004). We demonstrated higher amount of IGF-IR positive cells in malignant tumors than in nonmalignant ones. Similarly to the present results, in studies related to breast cancer in women, it was proved that most of the breast cancers had IGF-I receptors, which in particular referred to malignant tumors and to a lesser extent to nonmalignant lesions or normal glandular tissue (Happerfield et al. 1997, Shimizu et al. 2004, Koda et al. 2005a). Similarly, Queiroga et al. (2008) revealed that IGF-I concentration was higher in malignant canine mammary gland tumors comparing to nonmalignant lesions, but the authors used radio-immunoassay (RIA) and enzyme-immunoassay (EIA) techniques. On the other hand, Schnarr et al. (2000) presented opposite conclusions, which pointed an increase in IGF-IR expression in human duct carcinoma well-differentiated (Iº) and moderately-differentiated (IIº), whereas moderate decrease or lack of expression was found in poorly-differentiated carcinomas (IIIº).

It is well known that IGF-IR is an important link in proliferation and differentiation of the epithelial cells, and that is why it is considered as a cells proliferation marker. Since its right function provides the proper development, thus overexpression of the IGF-IR leads to the proliferation disturbances, which in consequence allows the neoplastic transformation. In the present study, higher activity of proliferation was shown in tumors of medium and high IGF-IR expression (Fig. 3). Similar data were obtained in studies concerning the breast cancer (Surmacz 2000, Yee et al. 2000, Yanochko et al. 2006). The results obtained may suggest a role of IGF-IR in carcinogenesis of the mammary gland in bitches.

Many of the references suggest that estrogens enhance IGF-I mitogenic activity and local synthesis of IGF-I in the breast cancer cells (Surmacz 2000, Oh et al. 2002, Koda et al. 2003), however until now there are no immunohistochemical studies concerning IGF-IR and ERh role in canine mammary gland tumors. Queiroga et al. (2008) found that estradiol acts synergistically with IGF-I stimulating epithelium proliferation towards malignant fenotype, however these results were obtained by RIA and EIA techniques. In the present study increased IGF-IR expression was observed in neoplastic cells showing high ERα, PR ex-

pression (Fig. 3). These results are in an agreement with some of the studies conducted in human medicine; in one of the works that employed IHC to label IGF-IR, a significant dependence between IGF-IR and ERα expression was demonstrated (Oh et al. 2002). The authors' conclusions pointed that increased IGF-IR expression can be related to the development of estrogen dependent breast cancer and this statement is currently accepted by other authors (Surmacz et al. 2000, Yee et al. 2000, Bradley et al. 2008). Data concerning IGFs correlation with progesterone receptor in breast cancer are poor and inconsistent. Only few contributions reported findings similar to those of the present study and showed the existence of "cross-talk" between IGF and PR (Katzenellenbogen et al. 1990). In veterinary medicine only Queiroga et al. (2008) proved the correlation between these markers. The authors suggested that progesterone induced local IGF-I and GH synthesis which may promote the development of these tumors as well as their malignancy.

IGF-IR acts in multiple directions and is not only limited to cells growth and differentiation. This receptor plays also an important role in apoptosis via the influence on *p53* gene and Bcl-2 family proteins expression (Surmacz 2000). The present results and those reported by other authors indicate that the presence of p53 protein is related to malignant histological type, high-grade and high proliferative activity of tumors, which is usually associated with poor prognosis (Davidoff et al. 1991, Lee et al. 2004, Rodo et al. 2008). This allow to conclude that determination of *p53* status may be useful in tumor grading and prognosis. Additionally, the present results suggest that IGF-IR overexpression is related to high p53 protein expression in canine mammary tumor cells. We suppose that this phenomenon is caused by increased *IGF-IR* gene expression in correlation to *p53* gene mutation. Some reports in human medicine concerning relationship between the IGF-IR expression and status *p53* gene suggest that this relation causes inhibition of apoptosis and leads to increased survival of neoplastic cells (Abramowitch et al. 2003).

In the present study, IGF-IR expression was positively correlated only with Bax protein expression. Similar results were obtained in human colonic carcinoma (Koda et al. 2004), while in breast cancer the authors did not show the influence of IGF-IR on Bcl-2 as well as Bax protein expression (Koda et al. 2005a). The results obtained suggest that IGF-IR activation leads to apoptosis induction, but still there is no clear answer which mechanisms are responsible for this process. It should be mentioned, that some reports confirmed a positive correlation between Bax, Bcl-xL proteins expression and insulin receptor substrate (IRS-1), which confirmed that IGF family proteins have an influence to maintain balance between pro- and anti-apoptotic factors in breast cancer (Koda et al. 2005).

On the basis of the present study it can be concluded that IGF-IR expression may be associated with the development and progression of canine mammary gland tumors (e. g. the relation between IGF-IR and p53), however it can not be classified into the significant prognostic marker category. In our opinion, steroid hormones have an influence on IGF-IR expression and IGF-IR expression initiates the proapoptotic pathway. However, the number of reports concerning the role of IGF-IR in canine mammary gland tumors is limited, it is suggested that further investigations in this area are needed, which could lead to the more final conclusions in the presented topic.

Acknowledgements

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